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Migrating Interstitial Cells Differentiate into Neurons in Hydra

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Interstitial cell migration has been observed numerous times in grafted hydra, but the extent to which graft injuries might stimulate the migration of otherwise nonmotile cells was unknown. The present study describes the migration and differentiation of vital dye-labeled interstitial cells in intact, normal hydra. Interstitial cells, stained with a fluorescent vital dye, migrated away from a labeled patch of ectodermal cells and subsequently were found throughout the body column. Shortly thereafter, labeled neurons began to appear among the migrating cells. The number of migrating interstitial cells remained constant over 5 days, whereas the number of labeled neurons increased. Labeled interstitial cells and neurons accumulated primarily in the head and peduncle, as observed in previous studies of short-term migration patterns in grafted hydra. The present study shows that migration of interstitial cells occurs in normal nongrafted hydra and that the accumulation patterns parallel results from graft experiments. The population of migrating cells appears to be limited to neuron precursors. © 1995 Academic Press, Inc.

INTRODUCTION

Distinct position-specific patterns of interstitial cell differentiation are maintained in the body column of hydra as the tissues of the animal grow and form new animals by budding (e.g., Bode and David, 1978). This phenomenon has been of considerable interest both from a patterning perspective and from a cell lineage approach. In hydra, the stem cells among the interstitial cell population give rise to three classes of somatic differentiation products (Fig. 1). Neurons are found throughout the ectoderm and endoderm of the animals, but differentiate primarily in the head and foot (e.g., David and Gierer, 1974; Heimfeld and Bode, 1981). Nematocytes differentiate in the ectoderm of the body column (e.g., Slautterback and Fawcett, 1959; David and Challoner, 1974) while secretory cells form in the endoderm of the gastric and head regions (e.g., Bode *et al.*, 1987). Because the stem cells are distributed along the length of the animal, a common view is that the stem cells make different commitment decisions in response to local environmental cues to generate the specific regional distributions of cells (Schaller, 1976; Bode and David, 1978; Yaross and Bode, 1978; Venugopal and David, 1981). Some interstitial cells are capable of migration, however (Tardent and Morgenthaler, 1966; Herlands and Bode, 1974a; Heimfeld and Bode, 1984a; Teragawa and Bode, 1990), suggesting that migration of committed but undifferentiated precursors could also contribute to position-dependent patterns of cell differentiation.

The extent of interstitial cell migration in normal, undisturbed hydra has been a matter of debate. Previously, interstitial cell migration had been observed in grafting studies and these migrating cells differentiated into nematocytes, neurons, and secretory cells (Herlands and Bode, 1974a; Heimfeld and Bode, 1984b; Bode *et al.*, 1987). Furthermore, multipotent stem cells also migrated in certain situations and could repopulate hydra consisting only of epithelial cells (e.g., Marcum and Campbell, 1978; Sugiyama and Fujisawa, 1978). In contrast to these results, Fujisawa *et al.*, (1990) showed that graft injuries stimulated interstitial cell migration, suggesting that migration was an artifact of injury of the animal. Another study analyzed the spatial growth pattern of interstitial cells and showed that the cells grew in contiguous patches, suggesting that the stem cells did not migrate freely in nongrafted animals (Bosch and David, 1990). Yet when a patch of cells in the body column of an intact animal was labeled with a vital fluorescent dye, DiI, migrating interstitial cells were observed a considerable distance from the patch (Teragawa and Bode, 1990). This provided direct evidence for the migration of interstitial cells in normal tissue in the absence of grafting.

In the previous DiI studies, animals were not observed

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long enough to determine fully the differentiation potential of the migrating cells. Here we extend our observations to determine what cell types are formed by the migrating interstitial cells. We examined animals for up to 7 days after staining and found that only neurons arose from the migrating interstitial cells. These results demonstrate that the migration of neuron precursors occurs in normal hydra and is a factor in establishing the high density of neurons found in the head and foot regions of the animal.

MATERIALS AND METHODS

Stock Cultures

All procedures utilized a strain of *Hydra vulgaris* originally isolated from Lake Zürich, Switzerland, by Dr. Pierre Tardent and maintained in a stock culture at Irvine for many years. Stock cultures were maintained at 18°C in hydra medium (Teragawa and Bode, 1990), fed three times a week with *Artemia* nauplii (San Francisco Bay Brand), and washed daily. Labeled animals were fed every 2 days.

DiI Labeling

DiI is a lipophilic dye that labels membranes of cells and has been used as a noninvasive marker in a number of other systems (Sims *et al.*, 1974; Honig and Hume, 1986; Selleck and Stern, 1991; Serbedzija *et al.*, 1992). A stock solution of DiI (1, 1′-dioctadecyl-3, 3, 3′, 3′-tetramethylindocarbocyanine perchlorate, Molecular Probes) was maintained as a saturated solution of DiI in 100% ethanol. Labeling solutions were freshly prepared from 1 part of the stock solution to 8 parts of an aqueous solution of 1% D-sorbitol (Sigma). Patches of tissue in whole, asexual hydra were labeled with a small amount of the dye injected directly into the midgastric region of the ectoderm with a glass micropipet, as in ink labeling of the ectoderm (Campbell, 1973). Samples in which the micropipet pierced the thin body wall and deposited label in the gut cavity were discarded, because in those cases the dye labeled the entire endodermal lining of the animal. Large animals both with and without buds were selected for labeling.

DiI can also label neurons by retrograde transport (Honig and Hume, 1989). To control for this possibility, some animals were treated with a 0.01 *M* solution of hydroxyurea (Sigma) for 2 days. This treatment reduces the number of rapidly dividing interstitial cells and nematoblasts in the tissue but does not eliminate the neuron population (Bode *et al.*, 1976; Sacks and Davis, 1979). After the treatment, the animals were allowed to recover for 3 days in hydra medium prior to labeling with DiI. The efficacy of the hydroxyurea treatment was monitored by macerating treated hydra (David, 1973) and by staining subsets of animals with 0.05% toluidine blue in 0.01 *M* Tris, pH 7.4 (Burnett, 1959), which stains interstitial cells and nematoblasts.

Observation of Labeled Hydra

Labeled animals were maintained in the dark prior to observation to minimize photobleaching of the label. Both living and fixed hydra were observed. Animals were fixed in 2% paraformaldehyde (Sigma) in PBS (0.01 *M* phosphate buffer, 0.15 *M* NaCl, pH 7.2). Over the course of the study, over 100 animals were examined, although not all of these were used for quantitative studies. Animals were viewed 1, 2, 5, and 7 days after labeling with conventional epifluorescence microscopy or with confocal microscopy using the Biorad Lasersharp MRC-500 imaging system. The DiI itself apparently exhibited no deleterious effect on cell differentiation in the labeled hydra, but unattenuated epifluorescent illumination of DiI-labeled, living hydra damaged the labeled cells, as previously observed in other systems (Honig and Hume, 1989), although the light exposure was not lethal to the hydra itself. Therefore, repeated observations on individual hydra were not performed in this study. Frozen sections of labeled hydra were examined by freezing fixed hydra in Tissue-Tek O. C. T. compound (Miles Diagnostics) and sectioning on a cryostat. Labeled grafted hydra were prepared as described in Teragawa and Bode (1990, 1991).

Migrating cells were defined as brightly and uniformly labeled cells that were found at least 100 μm beyond the edge of the labeled patch, although their exact locomotive activities were not known at the time of fixation. The cells had to be clearly separated from the patch by an area of tissue that was free of label. Cells were not counted if they were found in regions of high background staining of the epitheliomuscular cells (see Results).

Some labeled interstitial cells were observed that possessed long, filopodial processes and were presumably differentiating into neurons. These cells were counted as neurons if their processes were longer than the diameter of their cell body.

RESULTS

Nematocytes and Interstitial Cells Migrate

By injecting DiI into the ectoderm of the midgastric region, stained patches of tissue that spanned approximately one-sixth to one-fourth the length of the column and one-fifth to one-third the circumference of the column were produced. Frozen sections through the dye patch showed that the entire ectoderm became labeled, with parts of the ectoderm taking up label in the central portion of the injected area. The nuclear and plasma membranes and membranes within the cytoplasm were brightly labeled, permitting the identification of the various cell types which included epitheliomuscular cells, portions of the nerve net, interstitial cells, and nests of developing nematoblasts. The membranes retained a uniform staining pattern for approximately 5 days when viewed with conventional epifluorescence microscopy. Thereafter, the staining pattern became increasingly punctate although stained cells could still be

discerned. Therefore, for quantitative studies, we did not follow labeled cells beyond 5 days because of the increased difficulty in identifying large numbers of faintly labeled cells. Samples were examined for up to 7 days, however, for qualitative identification of differentiated cell types.

One day after labeling, large numbers of individual, DiI-labeled cells were observed a considerable distance from the labeled patch. Most of these cells were nematocytes and the remainder were interstitial cells. As previously described (Teragawa and Bode, 1990), the two cell types were readily distinguished by the distinct appearance of the nematocyst capsule in the nematocytes. Interstitial cells also had a more irregular cellular profile than did nematocytes and usually possessed a number of lamellipodial processes.

The migrating nematocytes were found primarily in the head and tentacle regions. This is consistent with previous observations showing that about 80–90% of these cells are destined for battery cells in the tentacles (Campbell, 1967b; Herlands and Bode 1974a, b; Bode and Flick, 1976). In contrast, the migrating interstitial cells were found throughout the body column except in the tentacles. Numerous cells were found concentrated in the head and peduncle regions of the hydra. Most of the migrating interstitial cells were single cells, although attached pairs of cells were also observed (Fig. 2). Although the depth of the migrating cells in the tissues was difficult to determine in wholemounts, nearly all of the nematocytes and interstitial cells appeared to be in the ectoderm, above the basal nerve net. A few interstitial cells did appear to be in the endoderm as they were in a focal plane beneath the nerve net.

Migrating Interstitial Cells Differentiate into Neurons

During the 7 days of observation after labeling with DiI, only two classes of labeled differentiation products, nematocytes and neurons, were observed at a distance from the labeled patch. Most neurons were ganglionic cells although a total of 8–10 sensory neurons were observed in head regions of the hydra. No labeled nests of nematoblasts or other cell types were observed far from the labeled patch.

Several divisions are required before a nematocyte precursor cell forms a nest of nematoblasts (Fig. 1), and it is possible that single nematocyte precursor cells may migrate, but their subsequent nests escape detection due to dilution of the DiI label. To test this possibility, the basal two-thirds of DiI-labeled hydra was grafted to the upper one-third of the body column of an unlabeled animal to see if labeled nests could be detected across the border. Animals were examined for labeled nematocyte precursors 2, 4, and 7 days after labeling and grafting. Only pairs of cells were observed 2 days after grafting (sample size, $n = 7$), but by 4 days after grafting, 22 labeled nests of 4 to 8 cells were seen ($n = 14$). Most of the labeled nests remained close to the original injected patch and did not move far into the unlabeled tissue, although 5 nests of 4 and 1 nest of 8 cells were found in the unlabeled head tissue. Nests of 4 and 8 cells also entered unlabeled buds. Seven days after grafting, 23 nests of 4 to 8 cells, and 3 nests of 12 to 16 cells were seen ($n = 8$). The increase in the number of larger labeled nests with time shows that the nematocyte precursors were progressing down the differentiation pathway at approximately their characteristic 1-day cell cycle time (David and Gierer, 1974). These results show that nests of 4, 8, and 16 cells retain the DiI label 7 days after labeling and therefore dilution of label is not the reason for the absence of migrating labeled nematocyte precursors. Rather, nematocyte precursors do not appear to migrate very far nor do they migrate in great numbers in grafts and in intact animals.

The migrating nematocytes arose from nests of nematoblasts in their final stages of differentiation (e.g., David and Gierer, 1974), so neurons thus appear to be the primary cell type to arise directly from a migrating interstitial cell precursor. Large numbers of labeled neurons were observed in this study; in the 55 animals examined quantitatively, over 650 total neurons were counted (Table 1). The labeled neurons were first observed 1 day after labeling and thereafter were seen in increasing numbers. Most neurons were found as single cells (Fig. 2A), although in the head and foot, where neuron density is high, 2 to 10 neurons were sometimes found near one another.

DiI is known to label neurons by retrograde transport (Honig and Hume, 1989), and neurons found far from the labeled patch could have been labeled by retrograde transport of DiI through neuronal processes that contacted the injected region, rather than by differentiation from labeled, migrating interstitial cells. To test if retrograde transport could produce the neuron labeling pattern we observed, the following control was performed. Hydra were treated for 2 days with hydroxyurea (HU), which reduces or eliminates the interstitial cell populations but has little effect on the neuron population (e.g., Sacks and Davis, 1979). In treated animals, the interstitial cell population dropped to 10% of normal. HU-treated animals were injected with DiI, fixed 2 days later, and examined for labeled interstitial cells and neurons. Labeled neurons were found in only 8 of 47 (17%) of the HU-treated animals compared to 13 of 16 (81%) normal animals. In the HU-treated animals, all of the labeled neurons and their processes were clearly separated from the injected patch by distinct regions of label-free tissue, and these neurons most likely arose from a few remaining labeled interstitial cells that survived the HU treatment. The strong decrease in neuron labeling in the HU animals shows that normal numbers of interstitial cells are necessary to produce the numbers of labeled neurons we observed. Little if any of the labeled neurons appear to arise from retrograde transport of label.

Observation of the large numbers of neurons in this study revealed a temporal pattern of morphological change which suggested the following stages in neuron differentiation. Beginning 1 day after labeling, migrating interstitial cells appeared that had two processes emerging from opposite ends of the cell (Fig. 2B). These cells had the appearance of bipolar neurons. The cell processes typically were aligned with the

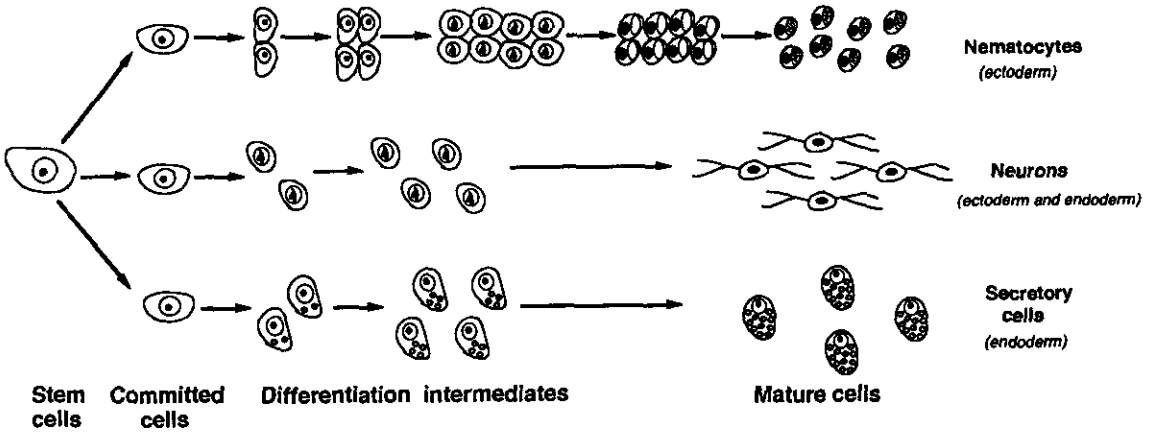

FIG. 1. A schematic of the interstitial cell lineage showing the three somatic differentiation pathways. Not all of the differentiation intermediates are indicated and the exact timing of commitment decisions remains controversial. There may be a variable number of cell divisions prior to differentiation. The locations of the differentiated cells in the endo- or ectoderm are indicated. Nematocytes differ from the other lineages in that they arise from syncytial nests of synchronously differentiating interstitial cells. Nematocytes migrate as mature cells, whereas only the differentiation intermediates of neurons and secretory cells are migratory.

muscle processes of the ectodermal epitheliomuscular cells. A few multipolar neurons (Fig. 2A) were observed on Day 1, but they began to appear in substantial numbers 2 days after labeling. These cells had the characteristic appearance of hydra ganglionic neurons (e.g., McConnell, 1932; Burnett and Diehl, 1964; Davis *et al.*, 1968). The numbers of bipolar cells remained constant at 3 to 6 per animal during the 5 days after labeling. In contrast, the multipolar cells continued to increase from 10% of all neurons on Day 1 to 57 and 87% on Days 2 and 5. The constancy of the numbers of bipolar cells and the continuous increase in the numbers of multipolar cells suggests that the bipolar cells are an intermediate stage in the ganglionic neuron differentiation pathway.

Regional Distribution of Migrating and Differentiating Cells

The numbers of interstitial cells that emigrated from the labeled patches and differentiated into ganglionic neurons were measured by counting labeled interstitial cells and neurons 1, 2, and 5 days after labeling. The average numbers of cells per animal were then calculated. As shown in Table 1, the average number of migrating interstitial cells remained fairly constant from day to day at 12 to 16 cells per animal. In contrast, labeled neurons accumulated in the tissue with time. The numbers of labeled neurons increased from 4 cells per animal 1 day after labeling to 24 cells per animal 5 days after labeling, an average increase of 5 neurons per day. As these numbers represent the differentiation of only those interstitial cells emerging from the DiI-labeled patch, the total number of neurons differentiating from interstitial cells in an entire hydra will be much larger.

In addition to counting the numbers of migrating and differentiated cells, the location of each of these cells was determined and assigned to one of six axial regions. The average numbers of cells per each region in an animal are shown in Fig. 3. The interstitial cells and neurons had similar regional distributions. One day after labeling, both cell types had a graded distribution throughout the body column with a maximum in the tentacle zone, the lower portion of the head. Two days after labeling, 70–75% of the cells were located near the ends of the body column at the head or foot of the animal; 41% of the interstitial cells were in the tentacle zone, and 28% were in the peduncle (Fig. 3A); and 43% of the neurons were in the tentacle zone, and 31% were in the peduncle (Fig. 3B). This bimodal distribution of cells was similar 5 days after labeling. Some interstitial cells do stay in the gastric region, however, and there give rise to about 25% of the labeled neurons (Fig. 3B). Relatively small numbers of interstitial cells and neurons were counted in the hypostome and basal disk, because of the smaller size of these tissue regions.

DISCUSSION

Migration of Neuron Precursors Is a Normal Feature of the Interstitial Cell System

As described earlier, grafting experiments provided ample evidence that interstitial cells could migrate (e.g., Tardent and Morgenthaler, 1966; Herlands and Bode, 1974a; Heimfeld and Bode, 1984a; Teragawa and Bode, 1990) and differentiate into all three classes (Fig. 1) of somatic products (Herlands and Bode, 1974a; Heimfeld and Bode, 1984b; Bode *et al.*, 1987; Marcum and Campbell, 1978). The DiI-injection procedure further demonstrated that migration of inter-

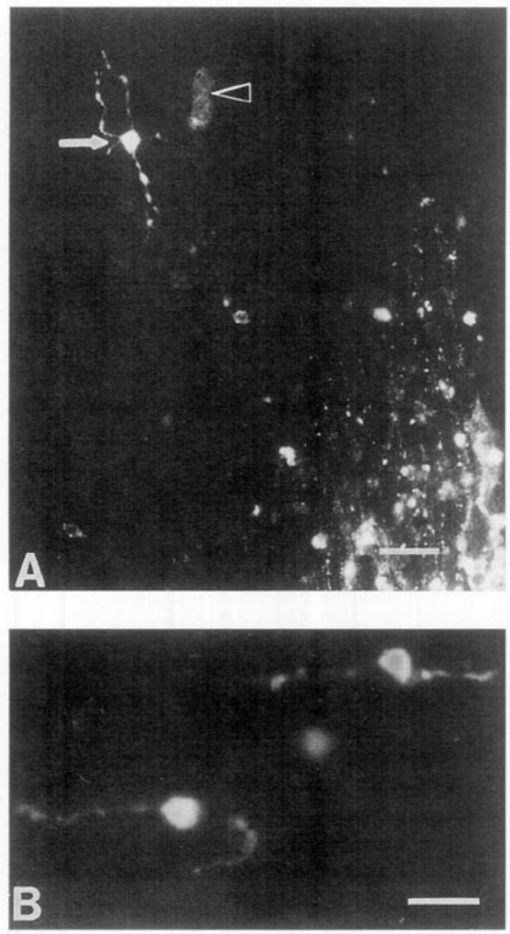

FIG. 2. DiI labeling of hydra. (A) Labeled products of migrating cells, 7 days after injection, confocal image. A pair of interstitial cells (arrowhead) and a labeled neuron (arrow) are seen in the upper gastric region a short distance away from the injected region at the lower right. Scale bar, 50 μm. (B) Two bipolar cells located in the peduncle, 2 days after labeling, epifluorescence image. Scale bar, 20 μm.

stitial cells normally occurs in intact hydra (Teragawa and Bode, 1990), but the present data show that only nematocytes and neuron precursors migrate. David and Hager (1994) also observed this result in their DiI studies. Grafting transiently increases the rate of interstitial cell migration (Fujisawa *et al.*, 1990) and comparison of the grafting and DiI studies suggests that some types of interstitial cells that normally do not migrate are induced to migrate by the effect of the grafting injury.

Had we observed substantial numbers of migrating stem cells, or nematocyte and secretory cell precursors, we should have seen (1) syncytial nests of labeled cells developing along the nematocyte pathway (e.g., Slautterback and Fawcett, 1959; David and Challoner, 1974), as about 30% of the interstitial cells normally enter this pathway (David and Gierer, 1974), and (2) DiI-labeled interstitial and secretory cells in the endoderm, as labeled ectodermal interstitial cells migrate to the endoderm to form the gland and mucous cells (Smid and Tardent, 1984; Bode *et al.*, 1987). Although a few labeled nests were observed, they were always close to or continuous with the injection spot and did not meet our criteria for migration (see Materials and Methods). A few interstitial cells appeared in the endoderm, but no consistent pattern of gland or mucous cell staining was detected in the large number of animals observed, in contrast to the highly consistent patterns of accumulation of labeled interstitial cells and neurons. It is possible that very small numbers of cell types other than neurons precursors may migrate and remain undetected as detection of labeled differentiation products depends upon visual recognition of cells in three-dimensional wholemounts. Such cells may not move far before differentiating and would be obscured by the patch of labeled tissue, or cells may migrate but fail to differentiate during the 7 days of observation. If such a cryptic population of cells does exist, it must be very small as even the scant number of sensory neurons (8–10 per 100 animals) was detected, and Table 1 shows there was no accumulation of undifferentiated interstitial cells over time.

Our results resemble results obtained in short-term grafting studies in that the graded distribution of migrating interstitial cells 1 day after labeling (Fig. 3) is similar in both the intact and grafted animals (Herlands and Bode, 1974a; Heimfeld and Bode, 1984a; Teragawa and Bode, 1990, Fig. 11). In both intact and grafted animals, cells preferentially accumulate in the apical head region of the hydra and are blocked from migrating into the tentacles (Teragawa and Bode, 1991). This similarity in cell distribution suggests that neuron precursors also make up the bulk of migrating cells in grafts as they do in intact animals. Grafting therefore does not appear to alter the accumulation pattern of the migrating neuron precursors. The results that we have seen from grafting studies of hydra may be an amplified version of events that occur normally during hydra development.

Migration of Neuron Precursors Contributes to Axial Patterns of Neuron Differentiation

The nerve network of hydra (Bode *et al.*, 1988; Bode, 1992), like the epithelial cells of the hydra body column, is

TABLE 1
Average Number of DiI-Labeled, Migrating Cells per Animal

Days after labeling	*N*	Migrating interstitial cells	Total neurons
1	24	15 ± 10	4 ± 3
2	12	12 ± 8	13 ± 6
5	19	16 ± 11	24 ± 14

Note. Values are means ± SD. Total neurons include bipolar and multipolar cells.

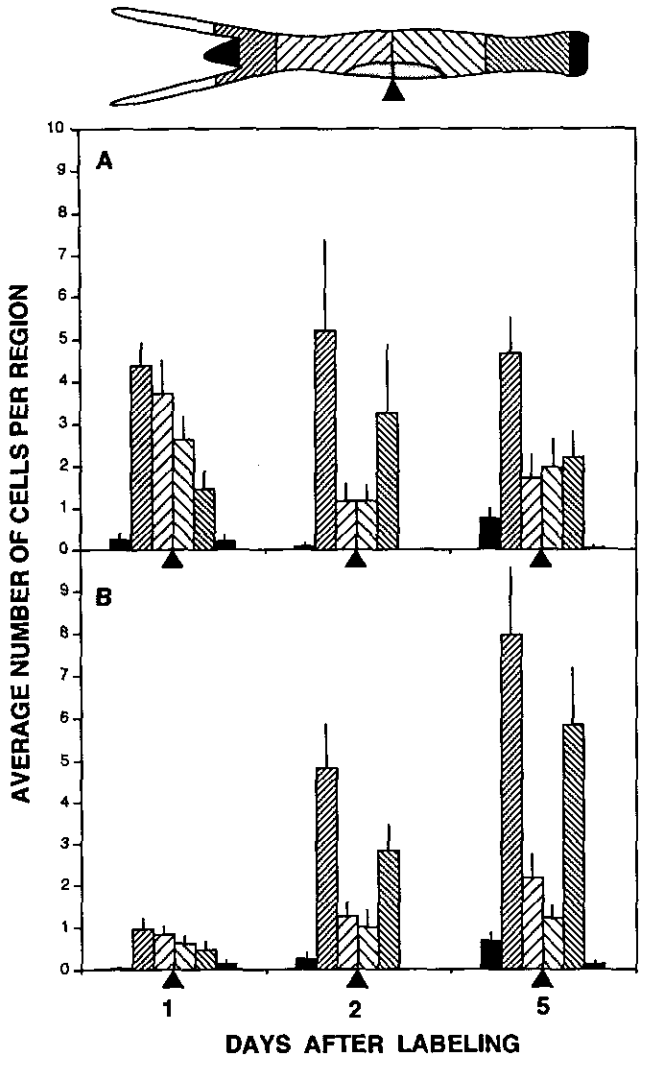

FIG. 3. Changes in the regional distribution of labeled interstitial cells and neurons. Shown are the average numbers of labeled interstitial cells (A) and neurons (B) found in various regions of the samples 1, 2, and 5 days after labeling. For simplicity, the numbers migrating into buds have been excluded and the totals thus differ slightly from the numbers in Table 1. The patterns of the bars correspond to the patterns of the hydra regions in the top diagram. The solid bars representing the hypostome are to the left. The solid triangles indicate the location of the DiI-labeled patch. Lines on the bars indicate standard errors of the mean. The regions were defined as (1) the hypostome, or upper part of the head surrounding the mouth; (2) the tentacle zone, or lower head region, where the tentacles emerge and including the proximal fifth of the tentacles; (3) the upper gastric region, below the tentacle zone and to the midpoint of the body column; (4) the lower gastric region, from the midpoint of the body column to the top of the peduncle; (5) the peduncle; and (6) the basal disk at the bottom of the hydra.

in a steady state of production and loss as cells are constantly displaced along the body column toward an extremity or into a bud during growth (Campbell 1967b, 1973). As this displacement occurs, the nonuniform densities of neurons throughout the body column are maintained by higher measured rates of neuron differentiation in the extremities (David and Gierer, 1974; Yaross and Bode, 1978; Heimfeld and Bode, 1981). Seventy-five percent of the DiI-labeled, migrating interstitial cells accumulate in the head and the peduncle (Fig. 3) and differentiate into neurons, and this result and earlier data obtained in grafting experiments (Heimfeld and Bode, 1984a; Fujisawa, 1989) show that the selective accumulation of migrating neuron precursors in these regions contributes to the higher rates of neuron differentiation in the extremities. Thus, position-specific commitment of the multipotent stem cells in the body column is not the only mechanism for generating the regional distributions of neurons. In addition to this mechanism, the migration of neuron precursors to specific locations can also help produce the observed distributions of neurons. Multipotent stem cells which produce migrating progeny may become committed to neuron differentiation in response to cues unrelated to position. For example, an internal stochastic process may govern neuron commitment as is postulated to occur during the commitment of stem cells in murine hemopoiesis (e.g., Ogawa, 1993). The commitment of stem cells to neuron differentiation could thus occur anywhere along the body column and selective migration and accumulation could redistribute the final products. A consequence of this view is that the migration of neuron intermediates into the extremities, but not the commitment of stem cells, is controlled by positional cues.

The present results alone cannot establish the actual commitment state of the neuron precursors, but they are consistent with the observations of David and Hager (1994), who found that neuron precursors could migrate, then divide one or sometimes two times prior to neuron differentiation. These authors suggest that their cells become both migratory and committed to neuron formation during S phase of the cell cycle. Although it is possible that there are uncommitted migrating cells that differentiate in response to positional cues in the head or foot, we also suggest that the migrating cells were precommitted to a neuronal fate prior to their arrival in an extremity, as even migrating cells that accumulated in the gastric region of the body column differentiated into neurons (Fig. 3), indicating that residence in an extremity was not a requirement for neuron differentiation. Our results differ from those of David and Hager in that they typically observed neurons differentiating in pairs in close proximity, whereas most of the neurons observed here appeared to differentiate as single cells (Fig. 2), although we did observe pairs of interstitial cells and groups of neurons. This variability may be a subjective difference in interpretation of the visual data, or it may indicate an interesting flexibility in the ability of cells in the neuronal lineage to proliferate prior to final neuron differentiation. Some cells may migrate as postmitotic precursors, while others may retain the ability to divide. The variation may be due to differences in the rapidity of tissue growth of the hydra.

In summary, a subset of the interstitial cell population migrates during the normal course of cell division and differentiation in hydra. The migratory cells appear to be restricted to neuron differentiation and accumulate preferentially in the head and peduncle regions of the hydra. The accumulation of the migrating neuron precursors toward the head and foot regions of the hydra shows that position-dependent migration of neuron precursors, in addition to position-dependent differentiation of multipotent cells, is a viable means of obtaining the concentrations of neurons observed in these regions of the hydra.

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